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Chemistry & Biology
Article

Analysis of Two Additional Signaling Molecules in *Streptomyces coelicolor* and the Development of a Butyrolactone-Specific Reporter System

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SUMMARY

γ-Butyrolactone bacterial hormones regulate antibiotic production and morphological differentiation in *Streptomyces* species. One γ-butyrolactone, SCB1, has been previously characterized in *Streptomyces coelicolor*. Here we report the characterization of two additional γ-butyrolactones, named SCB2 (2-[1′-hydroxyoctyl]-3-hydroxymethylbutanolide) and SCB3 (2-[1′-hydroxy-6′-methyloctyl]-3-hydroxymethylbutanolide), possessing an antibiotic stimulatory activity. To elucidate the specificity determinants of these ligands for the receptor protein, ScbR, 30 chemically synthesized γ-butyrolactone analogs were tested by utilizing the release of ScbR from DNA upon binding to a γ-butyrolactone, which can be detected by kanamycin resistance. The butyrolactone detection method developed here revealed that ScbR shows preference toward a ligand possessing a 7–10 carbon C-2 side chain, a C-1′-β-hydroxyl group, and a C-6′-methyl branch that coincides with SCB3. Moreover, this method was successfully used to screen for potential γ-butyrolactone producers from commercial-antibiotic-producing *Streptomyces.*

INTRODUCTION

Small extracellular signaling molecules called γ-butyrolactones are involved in the regulation of antibiotic production and, in some cases, morphological differentiation in the soil-dwelling *Streptomyces* (Takano, 2006). Regarded as bacterial hormones, the γ-butyrolactones are active at nanomolar concentrations and are thought to diffuse freely in and out of the cell. Twelve γ-butyrolactones, which have a 2,3-di-substituted γ-butyrolactone skeleton in common, have been identified to date. These have been classified into the following three groups based on their structural differences (Figure 1): (1) A-factor type, possessing a 1′-keto group; (2) virginiae butanolide (VB) type, possessing a 1′-α-hydroxyl group; and (3) IM-2 type, possessing a 1′-β-hydroxyl group (Choi et al., 2003). Early studies in *Streptomyces coelicolor* led to the partial elucidation of six γ-butyrolactone compounds (see Figure S1 available online; Anisova et al., 1984) but no biological function was determined in the producing strain. Takano et al. (2000) characterized the IM-2-type compound SCB1 from *S. coelicolor* that possesses antibiotic stimulatory activity, but does not have same structure as those identified by Anisova et al. (1984). In this study, two additional γ-butyrolactones, named SCB2 and SCB3, were successfully isolated from *S. coelicolor* and their chemical structures elucidated. Like SCB1, they also exhibit antibiotic stimulatory activity in the producing strain.

The published and most widely used assays for detecting the γ-butyrolactone signaling molecules are bioassays that detect antibiotic production (Hara and Beppu, 1982; Nihira et al., 1988; Sato et al., 1989; Takano et al., 2000). For example, in *S. coelicolor*, the SCB1 activity is determined by observing the induced production of the pigmented antibiotics actinorhodin and undecylprodigiosin (Takano et al., 2000). These bioassays, however, can be tedious and in some cases difficult to use because antibiotic production can be effected by indicator lawn concentration, medium conditions, and cultivation time. Furthermore, the γ-butyrolactones are produced in minute quantities and are difficult to isolate. Therefore, to study ligand specificity to the receptor and to detect novel γ-butyrolactones, a simple yet sensitive and reproducible detection method is necessary.

Previous studies revealed that the γ-butyrolactone receptor, ScbR, regulates the antibiotic production in *S. coelicolor* through repressing the transcription of its own gene and that of *cpkO*, a pathway-specific regulatory gene for the CPK antibiotic biosynthesis gene cluster (Takano et al., 2005 and Pawlik et al., 2007). The repression is abolished by the presence of the γ-butyrolactones, resulting in the transcription of the target genes. Based on these findings, we have designed and tested a new γ-butyrolactone detection method using ScbR as a sensor and the *cpkO* promoter coupled with a promoterless kanamycin resistance gene as a reporter to detect the γ-butyrolactones.

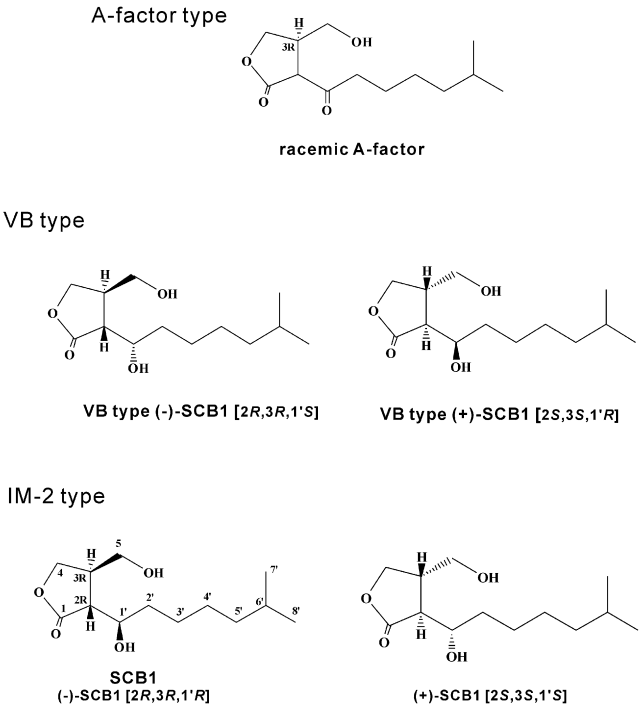


Figure 1. The Types of the γ-Butyrolactones and Their Examples

The three different types of the γ-butyrolactones are indicated and their examples from SCB1. Chemical structures of SCB1 [(−)-SCB1 (2*R*,3*R*,1′*R*)], its isomers [(+)-SCB1 (2*S*,3*S*,1′*S*)], VB type [(−)-SCB1 (2*R*,3*R*,1′*S*)], VB type [(+)-SCB1 (2*S*,3*S*,1′*R*)], and the racemic A-factor are shown, and the names of chemical structures are indicated below.

Synthetic analogs of A-factor were analyzed for their biological activity in *Streptomyces griseus*, the cognate host for A-factor (Khokhlov, 1991). Similarly, Nihira and coworkers chemically synthesized VB analogs (Nihira et al., 1988). These analogs were tested for their ability to induce virginiamycin in the VB cognate host, *Streptomyces virginiae.* These results from both groups indicate the specificity of the VB or A-factor receptor and the γ-butyrolactone analogs. However, a systematic analysis of chemically synthesized γ-butyrolactone analogs to the receptor protein, ScbR, from *S. coelicolor* has never been carried out.

Therefore, to elucidate the specificity determinant, we have synthesized 30 γ-butyrolactone analogs including two newly identified SCBs, SCB2 and SCB3. The biological activity of the analogs was measured using the novel kanamycin bioassay and affinity to ScbR was analyzed. Furthermore, we report the possible application of this assay to detect γ-butyrolactones produced by actinomycetes.

RESULTS

The Isolation and Determination of New SCBs from *S. coelicolor*

The structure of SCB1, a γ-butyrolactone in *S. coelicolor* M145 with antibiotic stimulatory activity, has previously been determined (Takano et al., 2000). High-performance liquid chromatography (HPLC) analysis indicates the presence of two further peaks possessing antibiotic stimulatory activity in *S. coelicolor* culture extracts, in addition to SCB1. These two minor peaks were further analyzed using an antibiotic bioassay described previously (Takano et al., 2000).

One HPLC peak fraction with a retention time (RT) very soon after SCB1 was applied to a C18 reverse-phase column and a bioactive peak with a RT of 24–25 min was detected (Figure 2A). This sample was subjected to 400 MHz ^{1}H-NMR for structure elucidation and showed that the structure might be similar to that of IM-2 C_8, especially because a triplet Me signal at 0.89 ppm indicated the presence of one terminal Me on a linear side chain, unlike the terminally branched side chain of SCB1 showing doublet Me signals (Figure S2). To further determine the structure, chemically synthesized racemic IM-2 C_8 (see Experimental Procedures) was used for comparison of the C18 HPLC retention time, which suggested the structure to be correct (Table 1). Furthermore, the bioassay of the synthetic IM-2 C_8 was active at similar concentrations to the natural SCB1 (data not shown). This compound was named SCB2 and the structure was determined as 2-(1′-hydroxyoctyl)-3-hydroxymethylbutanolide (Figure 2B).

The other minor HPLC peak fraction was also applied onto a C18 reverse-phase column and a bioactive peak with the RT of 41–45 min was detected (data not shown). Assuming that the lactone backbone is retained, the RT of 41–45 min is in between those of IM-2 C_8 (linear C8 side chain, 24–25 min) and IM-2 C_9 (linear C9 side chain, 54–56 min). Therefore the structure of the compound with this RT could be IM-2 C9-sec or IM-2 $C_{9\text{-i}}$.

To further test this hypothesis, the two racemic IM-2 C9-sec and IM-2 $C_{9\text{-i}}$ were chemically synthesized and the RT was determined on a reverse-phase HPLC. The chemically synthesized racemic IM-2 C9-sec eluted at 44–46 min whereas the racemic IM-2 $C_{9\text{-i}}$ eluted at 48–50 min (Table 1), suggesting that IM-2 C9-sec might be the structure of the second peak. This compound was named SCB3 and the structure was determined as 2-(1′-hydroxy-6′-methyloctyl)-3-hydroxymethylbutanolide (Figure 2C).

To further confirm the structure of SCB2 and SCB3, mass spectrometry (MS) and tandem MS were conducted using the high-accuracy Orbitrap MS on the synthesized SCB1, SCB2, SCB3, IM-2 $C_{9\text{-i}}$ and the ethyl acetate extract of supplemented minimum medium solid (SMMS)-grown *S. coelicolor* M145. The RT for the synthesized SCB1, SCB2, SCB3, and IM-2 $C_{9\text{-i}}$ were 40.33, 41.12, 44.21, and 49.35/57.57, respectively (Table 1). In the M145 ethyl acetate extract, RTs that correspond to the mass equivalent to SCB1 [$(M+H)^+$ 245.17], SCB2 [$(M+H)^+$ 245.17], and SCB3 [$(M+H)^+$ 259.19] were similar but somewhat earlier than the synthetic compounds (Table 1). This might be due to the impurity in the M145 ethyl acetate extracts. However, a mass corresponding to SCB3 [$(M+H)^+$ 259.19] was not detected at the same RT as IM-2 $C_{9\text{-i}}$. This strongly confirms the previous analysis and that the structure of SCB3 is indeed 2-(1′-hydroxy-6′-methyloctyl)-3-hydroxymethylbutanolide. The MS and tandem MS spectra of the captured molecules in the M145 ethyl acetate extract show high similarity to the synthesized SCB1 and SCB2 [both have $(M+H)^+$ 245.17] and also to SCB3 [$(M+H)^+$ 259.19] spectra that include the mono- or di-dehydrated structures of SCB1, SCB2, or SCB3, confirming the production of these three butanolides in M145.

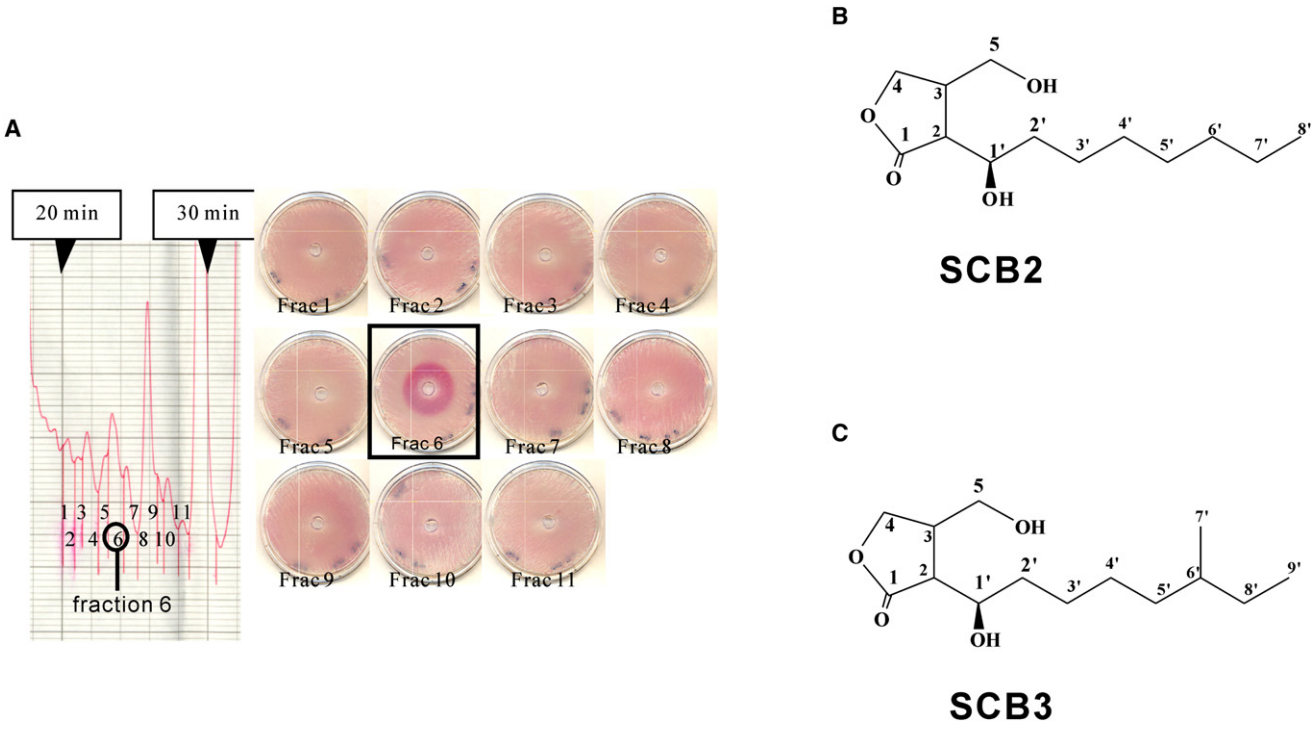


Figure 2. HPLC Profile and Antibiotic Bioassay for SCB2 and the Chemical Structure for SCB2 and SCB3

(A) HPLC profile of the M145 ethyl acetate extract is shown with the corresponding fractions analyzed for γ-butyrolactones by antibiotic bioassay. Fraction 6 gave bioactivity and was further purified and shown to be SCB2.

(B) The elucidated chemical structure of SCB2 [2-(1′-hydroxyoctyl)-3-hydroxymethylbutanolide].

(C) The elucidated chemical structure of SCB3 [2-(1′-hydroxy-6′-methyloctyl)-3-hydroxymethylbutanolide].

Synthesis of γ-Butyrolactone Analogs for Use in Structure-Activity Studies

The newly identified SCBs differ in the nature of the C-2 side chain compared with the previously identified compound SCB1. To determine the specificity of these ligands to the receptor, further racemic analogs, IM-2 type, VB type, and A-factor type were chemically synthesized with different side-chain length and branching. Thirty different analogs can be grouped into four types based on structure: (1) SCB1 isomers: IM-2 type: (−)-SCB1 [2*R*,3*R*,1′*R*], (+)-SCB1 [2*S*,3*S*,1′*S*], VB type: (−)-SCB1 [2*R*,3*R*,1′*S*], (+)-SCB1 [2*S*,3*S*,1′*R*]; (2) IM-2-type analogs possessing a C-1′-β-hydroxyl group with various linear and branched C-2 side chains; (3) a VB-type series possessing a C-1′-α-hydroxyl group with various linear and branched C-2 side chains; and (4) A-factor type series possessing a C-1′-keto group. The chemical structures are shown in Figures 1 and 4.

A New γ-Butyrolactone Detection Assay Using Kanamycin Resistance

To test the activity of the chemically synthesized analogs, the previously published antibiotic bioassay based on the precocious production of colored antibiotics could have been utilized (Takano et al., 2000). However, this method is not always easy to score because the production of the colored antibiotics can vary with time, density of the inoculum used for the indicator lawn, and batch of media used. Therefore, a new reporter system that is both easier to perform and gives a clearer measure of γ-butyrolactone activity was developed.

ScbR, the γ-butyrolactone receptor, which has been shown to be the only receptor that can recognize the γ-butyrolactones to promote antibiotic stimulatory activity, binds to its own promoter region and to that of *cpkO*, a pathway-specific regulatory gene for the CPK cluster, and thereby represses the transcription (Takano et al., 2005 and Pawlik et al., 2007). In the

Table 1. Retention Time of SCB1, SCB2 and SCB3 Chemically Synthesized or Extracted from M145 Analyzed by HPLC or by Nano-LC-Orbitrap MS

	Retention Time (min)	
Compound	HPLC (C18 RP Column)	Nano-LC-Orbitrap MS
Chemically synthesized		
SCB1	23–24	40.33
SCB2 (IM-2 C_8)	25–27	41.12
SCB3 (IM-2 C9-sec)	44–46	44.21
IM-2 C_{9-i}	48–50	49.35/57.57
M145 ethyl acetate extract		
SCB1	22–23	39.30
SCB2 (IM-2 C_8)	24–25	40.20
SCB3 (IM-2 C9-sec)	40–48	43.97

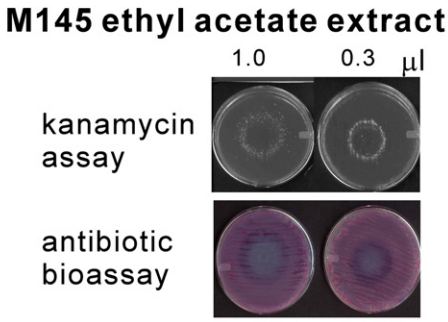
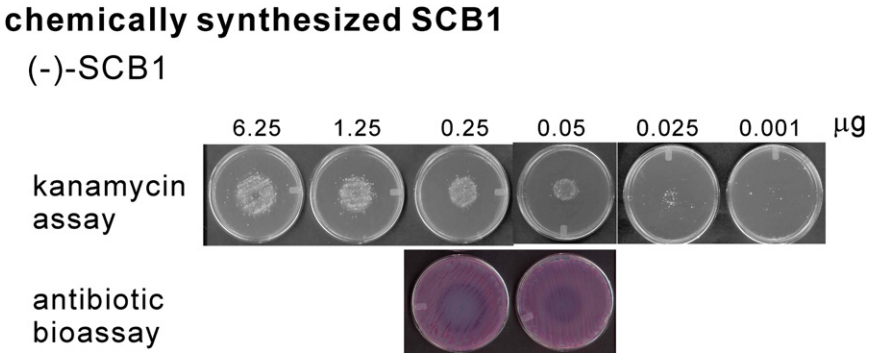


Figure 3. The Kanamycin Bioassay and the Antibiotic Bioassay Results of the M145 Ethyl Acetate Extract and the Chemically Synthesized SCB1

The M145 ethyl acetate extract and the chemically synthesized SCB1 were spotted onto a lawn of LW16::pTE134 on DNAgar plates containing 5 μg/ml kanamycin, or on a confluent lawn of M145. The amount of the extract and the SCB1 spotted is indicated above the plates. The plates were incubated at 30°C for 72 hr.



presence of γ-butyrolactones, ScbR binds to the γ-butyrolactones and relieves DNA binding, thus allowing the target genes to be transcribed (Takano et al., 2005). Utilizing these properties of ScbR, a reporter system comprising a copy of *scbR* controlled by its own promoter and the *cpkO* promoter complete with the ScbR binding site fused to a promoterless kanamycin resistance gene in the conjugative and integrating vector pIJ82 was constructed. This plasmid, named pTE134 (Figure S3), was introduced into *S. coelicolor* LW16 (a *scbA* and *scbR* double deletion mutant, see Experimental Procedures). LW16 does not produce any γ-butyrolactones with antibiotic stimulatory activity as determined by the antibiotic bioassay (data not shown). Therefore, in LW16, the ScbR resulting from pTE134 represses the *cpkO* promoter and the strain remains sensitive to kanamycin. In the presence of γ-butyrolactones, however, ScbR repression is relieved and the strain becomes kanamycin resistant. This method, therefore, was named the kanamycin bioassay and can be used to detect γ-butyrolactones that can bind to ScbR.

A confluent lawn of *S. coelicolor* LW16::pTE134 spores was plated onto Difco Nutrient Agar (DNAgar) plates containing 5 μg/ml kanamycin and spotted with synthesized SCB1 (6.25–0.001 μg) and the M145 ethyl acetate extract (1 and 0.3 μl) at the time of inoculation. After incubation, the kanamycin-resistant colonies grew around the spot where the γ-butyrolactones had diffused (Figure 3). The same samples, the SCB1 and M145 ethyl acetate extract, 0.25 and 0.05 μl and 1.0 and 0.3 μl, respectively, were used for the antibiotic bioassay and also gave precocious antibiotic production (Figure 3). To determine the lowest working concentration detectable by the kanamycin bioassay, SCB1 was added into the agar, instead of spotting on top of the agar. This verified the minimum concentration to be the same as the antibiotic bioassay reported previously (Takano et al., 2000), 256 nM SCB1, suggesting that both bioassays share the same detection sensitivity toward SCB1.

The kanamycin bioassay was used to compare the specificity of ScbR toward known natural γ-butyrolactones (Table 2). ScbR shows the highest affinity toward the naturally produced γ-butyrolactones in its cognate host, SCB1 and the racemic SCB3, with a 2-fold stronger activity than the racemic SCB2. The SCB1 used here is a pure stereochemical compound compared with the racemic mixtures. The (+)- stereochemical compound, which is present in the racemic mixture, showed 250-fold less activity in the case of SCB1 (Table 2), indicating that these stereochemical compounds for the other γ-butyrolactones might also have almost no activity and can be disregarded. Therefore, the activity for the racemic mixtures is approximately half for SCB2 (which possesses one chiral carbon) and a quarter for SCB3 (which possesses two chiral carbons) of what is observed. Taking this into account, the activity can be recalculated to: SCB1 = 0.025, SCB2 = 0.025, SCB3 = 0.006 μg. In conclusion, SCB3 has the highest affinity toward ScbR, followed by SCB1 and SCB2 (Table 2 and Figure S5).

The diameters of the halo detected with 6.25, 1.25, 0.25, 0.05, and 0.025 μg SCB1 are 3.5, 3, 2.7, 2, and 1 cm, respectively (Figure 3). This shows that the size of the growth halo increases with increasing amounts of the natural SCB1. However, the relationship is not linear but shows a sigmoidal curve, and therefore it is difficult to determine the concentration of samples by analyzing the size of the growth halo.

The IM-2-type γ-Butyrolactone Analog with a Long C-2 Side Chain Shows the Highest Specificity in the Kanamycin Bioassay

The chemically synthesized structural analogs of SCBs were tested for their binding specificity toward ScbR according to the kanamycin bioassay results. The activity measurement taken was the minimum amount of compound required for growth, as determined by finding the lowest amount of compound capable of inducing a visible halo of growth in the bioassay (Table 2).

Table 2. Minimum Concentration of γ-Butyrolactone Analogs that Show Kanamycin Resistance

Analogues	IM-2 Type (μg)	VB Type (μg)	A-Factor Type (μg)	SCB1 Isomers (μg)
C_2	-	NA	NA	NA
C_3	-	NA	NA	NA
C_4	-[a]	NA	-	NA
C_5	-	-	-	NA
C_6	12.5	-[b]	NA	NA
C_7	0.25	6.25[c]	NA	NA
C_8	0.05[d]	1.25	NA	NA
C_9	0.25	0.25	NA	NA
C_{10}	1.25	1.25	NA	NA
C_{11}	6.25	-	NA	NA
C9-sec	1.25	12.5	NA	NA
$C_{9\text{-}i}$	0.25	0.25	NA	NA
C9-sec	0.025[e]	1.25	NA	NA
A-factor	NA	NA	1.25[f]	NA
(−)-SCB1	NA	NA	NA	0.025[g]
(+)-SCB1	NA	NA	NA	6.25[h]
VB type (-)-SCB1	NA	NA	NA	6.25[h]
VB type (+)-SCB1	NA	NA	NA	6.25

All compounds are a racemic mixture apart from the four SCB1 isomers. Minus sign indicates no growth halo on the plate (the highest used amount is 31.25 μg, except for VB C_6, where 62.5 μg was used); NA, not applicable (this analog is unavailable). The superscript letters a–g indicate the natural γ-butyrolactones.

[a] IM-2.
[b] VB-C.
[c] VB-D.
[d] SCB2.
[e] SCB3.
[f] A-factor.
[g] SCB1.
[h] The growth halo was not clear.

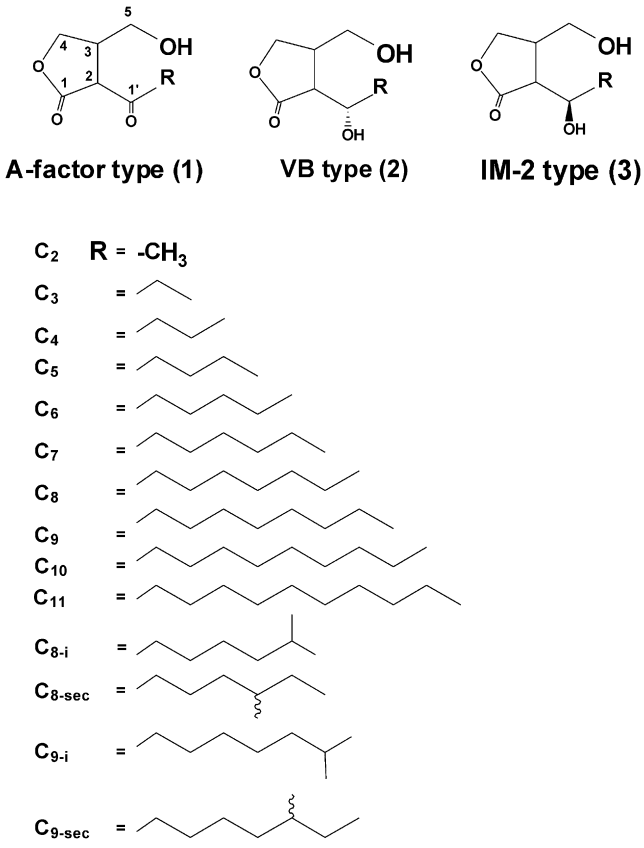


Figure 4. The Chemical Structure of the Synthesized γ-Butyrolactone Analogs

The chemical skeletons of γ-butyrolactones: (1) A-factor type, a C-1′-keto group; (2) VB type, a C-1′-α-hydroxyl group; and (3) IM-2 type, a C-1′-β-hydroxyl group. Linear and branched alkyl side-chain substitutes (R) at C-2 along with their names are specified below.

Racemic A-factor (6.25, 1.25, and 0.25 μg) was tested and the minimum amount required for growth was 1.25 μg (Figure S4). No growth was observed with racemic A-factor with a shorter linear C-2 side chain, C4 or C5 (Table 2).

Twenty-seven chemically synthesized SCB1 isomers, racemic IM-2-type, racemic VB-type γ-butyrolactone analogs were also tested using a range from 62.5 μg to 0.01 μg (Table 2, Figure 4). Consistent with the previous results (Takano et al., 2000), among the four pure SCB1 isomers (−)-SCB1, (+)-SCB1, VB type (−)-SCB1, and VB type (+)-SCB1, representing the four different configurations of SCB1, (−)-SCB1 showed the highest activity, the minimum 0.025 μg being 250-fold lower than with the other three analogs (Figure 1 and Figure S5). As also reported previously (Takano et al., 2001), ScbR showed a higher specificity toward the IM-2-type compounds possessing a 1′-β-hydroxyl group, rather than the VB type, which have a 1′-α-hydroxyl group (Table 2).

The activity of the chemically synthesized racemic IM-2 type analogs with varying lengths of the linear C-2 side chain was tested. Those that possessed a length of 6 to 11 carbons had the most specificity (Figure S6). No growth at all was detected with the analogs possessing 5 or fewer carbons using up to 31.25 μg compound. Because the maximum carbon chain length tested was C11, the activity beyond this length is not known. The most active among the IM-2-type analogs was the racemic SCB2 (IM-2 C_8, 0.05 μg), which possesses an 8 carbon C-2 side-chain length (Table 2).

Similarly, the specificity of the chemically synthesized racemic VB type analogs with varying lengths of the linear C-2 side chain was tested, and the 7 to 10 carbons had the highest specificity (Figure S7). No growth was detected using 62.5 μg racemic VB C_6 (racemic VB-C, data not shown).

The different branching positions at the same C-2 side-chain length within the IM-2-type and VB-type analogs were also compared. The pure SCB1 [(−)-SCB1], possessing a C-6′-methyl group, is 50-fold more active than the racemic IM-2 C8-sec, possessing a C-5′-methyl group, and 10-fold more active than the racemic IM-2 C_7, possessing a linear C-2 side chain. Furthermore, the racemic SCB3 (IM-2 C8-sec), possessing C-6′-methyl group, is 10-fold more active than the racemic IM-2 $C_{9\text{-}i}$, possessing a C-7′-methyl group, and 2-fold more active than the racemic SCB2 (IM-2 C_8), possessing a linear C-2 side chain (Table 2 and Figure S8). However, the VB-type analogs with a branched side chain did not show a significant difference (<6-fold) as seen in the IM-2 type compounds (Table 2 and Figure S8).

Table 3. The *Streptomyces* Species from the Tübingen Collection that Were Tested for γ-Butyrolactone Production

Strain	Antibiotic	Growth on Plates	Growth Halo
Streptomyces antibioticus ETH 6143 (Tü 2)	Cinerubin	O	–
Streptomyces antibioticus ETH 6677 (Tü 4)	Angolamycin	X	–
Streptomyces olivaceus ssp. *Atratus* (Tü 5)	Narbomycin	X	+
Streptomyces griseus ETH4289 (Tü 19)	Streptomycin	O	–
Streptomyces tendae ETH11313 (Tü 21)	Carbomycin	O	–
Nocardia brasiliensis ETH27413 (Tü 69)	Ferrioxamin E	O	–
Streptosporangium roseum ETH28349 (Tü 74)	Unknown	X	–
Streptomyces mediteranei (Tü 75)	Rifomycin	X	+
Microellobosporia cinerae ETH28583 (Tü 76)	Unknown	X	–

Tü 2–Tü 76 indicate strain number of the Tübingen strain collection. X represents strains that grew well on the SMMS and SMMS supplemented with yeast extract; O, strains that barely grew on the SMMS and SMMS supplemented with yeast extract; –, no growth halo on the plate; +, growth halo on the plate.

A summary of the minimum amount required for growth on kanamycin for the chemically synthesized compounds is shown in Table 2.

Identification of Two New Possible γ-Butyrolactone-Producing Actinomycetes Using the Kanamycin Bioassay

The kanamycin bioassay was used to identify possible γ-butyrolactone producers from among nine actinomycetes species obtained from the Tübingen strain collection (Table 3). These nine species were grown on SMMS for 3 days (Figure S9), and γ-butyrolactones were isolated by ethyl acetate extraction (see Experimental Procedures). Some species (*Streptomyces antibioticus* ETH 6143, *Streptomyces griseus* ETH4289, *Streptomyces tendae*, and *Nocardia brasiliensis*) did not grow well on SMMS or on SMMS supplemented with 5% yeast extract. Nevertheless, all strains were isolated and tested. Extracts from two of nine species, *Streptomyces olivaceus* ssp. *atratus* and *Streptomyces mediteranei*, which are producers of narbomycin and rifomycin, respectively, had an ability to induce a growth halo in the bioassay (Figure S9), suggesting that these two species produce compounds, most likely to be γ-butyrolactones, which have affinity toward ScbR.

DISCUSSION

Multiple γ-Butyrolactones Produced in *S. coelicolor*

Two additional signaling molecules, SCB2 and SCB3, from *S. coelicolor* were identified and both exhibit a similar lactone structure to the previously identified compound SCB1, as well as the same IM-2 type stereospecificity. The difference in structure was the C-2 side-chain length with a linear C8 for SCB2 and a branched C9 for SCB3. SCB2 did not correspond to any of the six γ-butyrolactones identified previously; however, because the chirality of SCB3 was not assigned, there is a possibility that it has the same structure as AcI1a (Figure S1; Anisova et al., 1984). Both newly identified compounds induce precocious antibiotic production in an *S. coelicolor* bioassay, and the highest affinity toward ScbR was shown by SCB3 followed by SCB1 and SCB2 as determined by the kanamycin bioassay.

The γ-butyrolactones from *S. coelicolor* are synthesized by ScbA, which is a protein also conserved in the A-factor and VB-producing *Streptomyces* (Hsiao et al., 2007 and Kato et al., 2007). The *S. coelicolor scbA* deletion mutant does not produce any γ-butyrolactones with antibiotic stimulatory activity (Takano et al., 2001), indicating that SCB1, SCB2, and SCB3 all are synthesized by ScbA. One possible explanation for the different side-chain structures of the three signaling molecule might be due to the precursors used in the biosynthesis, this suggests a somewhat relaxed substrate specificity for ScbA. From the proposed biosynthesis pathway of A-factor in *Streptomyces griseus*, a dihydroxyacetone phosphate and a β-keto acid derivative is condensed by the ScbA homolog and further reduction and dephosphorylation result in A-factor (Kato et al., 2007). The β-keto acid derivatives might be provided either by a dedicated biosynthesis pathway or by scavenging an intermediate from fatty acid metabolism. We have extensively analyzed the *S. coelicolor* genome and have been unable to identify a dedicated biosynthesis pathway capable of providing the β-keto acid derivatives. It is therefore seems likely that the fatty acid metabolism intermediates are the precursors for SCB biosynthesis. *Streptomyces* produce both linear and branched fatty acids (Han et al., 1998), which correlate to the C-2 side-chain differences observed in this work. The same hypothesis has been suggested by Kato et al. (2007).

Interestingly, *S. coelicolor* is not the only multiple γ-butyrolactone-producing strain to have been reported. Five different virginiae butanolides (VB-A to VB-E) were identified from *Streptomyces virginiae* (Yamada et al., 1987 and Kondo et al., 1989), and two active factors were isolated from *Streptomyces bikiniensis* (Gräfe et al., 1983). In addition, γ-butyrolactones from 11 *Streptomyces* species were analyzed by HPLC (Hashimoto et al., 1992), and only 3 species produced a single γ-butyrolactone, whereas the others produced two to four different compounds. It will be interesting to determine what the exact roles of these different γ-butyrolactones are in their producing strains.

The New γ-Butyrolactone Detection Reporter System

The kanamycin bioassay was successfully applied as a sensitive γ-butyrolactone detection tool in *S. coelicolor.* It utilizes the *cpkO* promoter, which is tightly controlled by ScbR and is fused to a kanamycin-resistance cassette along with the γ-butyrolactone receptor, ScbR, as a sensor. The high kanamycin sensitivity in the absence of γ-butyrolactone is due to the specific and high affinity of ScbR to the *cpkO* promoter resulting in the tight repression of the gene. Some spontaneous resistant mutants do arise, especially with low concentrations of kanamycin (Figure S5). To obtain optimal sensitivity of the assay, kanamycin

concentrations should be systematically varied to be able to monitor the resistant growth without any background.

The kanamycin bioassay has the same minimum active concentration for SCB1 as with the antibiotic bioassay (256 nM, data not shown), which is reasonable considering that both methods use ScbR as the sensor. However, when recognizing the non-cognate γ-butyrolactones, the kanamycin bioassay was much more sensitive than the antibiotic bioassay. Only 50-fold higher concentration of A-factor, compared with SCB1, was required to give activity with the kanamycin bioassay, whereas the antibiotic bioassay required 100-fold higher concentration (Takano et al., 2000).

The kanamycin bioassay is also more sensitive than other *Streptomyces* γ-butyrolactone assays when detecting the non-cognate signaling molecules. The A-factor bioassay required 3×10^3-fold higher concentration of VB (non-cognate signal) than A-factor by the streptomycin cosynthesis method using a streptomycin nonproducing mutant, *S. griseus* HH1 (Miyake et al., 1989). A concentration of A-factor or IM-2 (non-cognate signals) that was 1.7×10^4-fold higher than VB was required to trigger virginiamycin production in a liquid culture of *S. virginiae* (Hashimoto et al., 1992; Nihira et al., 1988), and a concentration of VB-A (non-cognate signal) that was 8.3×10^4-fold higher than IM-2 was required to induce the blue pigment production in liquid cultures of *Streptomyces lavendulae* FRI-5 (Hashimoto et al., 1992). This might reflect the higher sensitivity of the kanamycin bioassay because it is directly measuring the binding affinity of the ligand to the receptor and not detecting antibiotic production, which is the end product of the signaling cascade and might have other unknown influences. However the higher sensitivity can also lead to background kanamycin resistance observed in some cases, which can be due to perturbations and noise. In conclusion, this new assay is much clearer than the antibiotic bioassay, is simple to use, and directly exhibits the relationship between ScbR and the ligands.

Similar repressor-inducer reporter systems are available. Tahlan and coworkers have developed a potential screening method to detect ligands isolated from *S. coelicolor* that bind to tetracycline-inducible repressor (tetR)-like regulators from *S. coelicolor* using *Escherichia coli* as a host (Tahlan et al., 2008). The tetR-operator regulation system from *E. coli* transposon Tn*10* has been widely used as a tool in many organisms including *Streptomyces* (Stebbins et al., 2001; Rodríguez-Garcia et al., 2005). However, this is the first reporter system using a γ-butyrolactone receptor to detect the signaling molecules in *Streptomyces*.

ScbR Ligand Specificity

From our data, the chemical analogs with a shorter C-2 side-chain length than 8 (for the IM-2 type) or 9 carbons (for the VB type) resulted in a lower binding affinity than the SCBs and must have at least 6 (for the IM-2 type) or 7 (for the VB type) carbons and no more than 10 (for the VB type) carbons for the C-2 side-chain length to bind to ScbR. We do not have ligands with more than 11 carbons at the C-2 side chain to conclude the limit of the longest C-2 side-chain length for the IM-2 type. The IM-2-type analog with 9 carbons for the C-2 side-chain length with branching at C-6′ position showed the best affinity than the branching at C-7′ position and no branching, in this order. We have therefore shown for the first time, that the branching at the C-6′ position is the preferred ligand to bind to ScbR. In conclusion, the γ-butyrolactone structure with the highest affinity to ScbR is as follows: (1) IM-2 type configuration, (2) C-2 side-chain length of C7 or C8, (3) branching at the C-6′ group.

Twenty-one synthetic analogs of A-factor with varying lengths of either linear or branched C-2 side chains or different substitutes at C-3 position were analyzed for their biological activity in *S. griseus*, the cognate host for A-factor (Khokhlov, 1991). None of these analogs gave significant activity compared with A-factor except when the C-3 position was substituted for acylated products. The A-factor type analogs with one carbon longer or shorter than C8 all with a methyl residue at the end of the chain resulted in a 10-fold lower activity compared with A-factor (Khokhlov, 1991). Similarly, Nihira and coworkers chemically synthesized 41 VB analogs, varying the C-2 and C-3 stereochemistry to include the A-factor and *cis* and *trans* VB type of γ-butyrolactones as described above, and also varying the C-2 linear side-chain length up to C10, and different substitutes for the C-3 position (Nihira et al., 1988). These analogs were tested for their ability to induce virginiamycin in the VB cognate host, *S. virginiae.* The *cis* VB-type compounds with C7 and C8 linear side chain was most active whereas the *trans* VB type compounds with the equivalent side-chain length gave 12.5-fold less activity. The C9 A-factor type compound was the only A-factor analog to give the highest activity, which was 1250-fold less compared with the *cis* VB-type C7 compound, thus demonstrating the importance of the C-1′ free hydroxy group.

For all three *Streptomyces* γ-butyrolactone receptors that were tested using synthetic analogs, the length of the C-2 side chain is preferred to be C7 or C8. Furthermore, none exceeded in their binding specificity toward the receptor than the naturally produced γ-butyrolactones. To obtain chemical ligands that have better binding properties than the natural compounds, other structural substitutes than that of the C-2 side-chain length or branching will be interesting to test. Three-dimensional crystallization analysis of the receptor with the various γ-butyrolactones might give valuable information on further binding specificities and possible new structural substitutions.

Potential Application for the Kanamycin Bioassay

In the screening for possible new γ-butyrolactone producers (Table 3 and Figure S9), two of the nine tested strains from the Tübingen strain collection were able to produce compounds that could be recognized by ScbR. These strains have not been shown previously to produce any γ-butyrolactones. The extracts from the other strains did not show γ-butyrolactone-induced kanamycin-resistant growth, but this might be due to the unsuitable growth conditions used for the preparation of the extracts because in some cases the strains did not grow efficiently. It is also possible that the extraction of the γ-butyrolactone was not at the appropriate time of growth, because these compounds are known to be produced in a growth-dependent manner (Takano, 2006). Therefore, optimizing the suitable medium for growth and altering the timing for the extract preparation might assist in the detection of other γ-butyrolactones from different strains. This result, together with the higher sensitivity toward the γ-butyrolactones than the commonly used

bioassay, proves the feasibility of the kanamycin bioassay as a powerful tool for large-scale screening of γ-butyrolactone producers.

SIGNIFICANCE

The significant conclusion from this work is the characterization of two further γ-butyrolactones from *S. coelicolor* and the identification of the γ-butyrolactone receptor (ScbR) specificity determinant by use of a novel small signaling molecule reporter system analyzing both natural and synthetic ligands. This is the first report where many synthetic γ-butyrolactone analogs were tested systematically to compare their binding specificity to ScbR. The favored ligand consists of an IM-2-type β-hydroxyl group on the C-1′ position, with a long (7–10 carbons, with C8 representing the best) C-2 side chain. Surprisingly, we have been able to show for the first time the importance of a methyl branch at the C-6′ position of the C-2 side chain. This preference was also confirmed because the newly characterized γ-butyrolactone, SCB3, follows the ScbR ligand preference and has the highest affinity compared with the two other cognate signaling molecules. Understanding the ligand specificity determinant will allow further potential design of new compounds to either inhibit or to promote antibiotic production by controlling ligand binding to the γ-butyrolactone receptor. The new sensitive reporter system can be applied to detect γ-butyrolactones that are only produced in small quantities and can be difficult to detect. We have demonstrated this use by identification of two new commercial-antibiotic-producing *Streptomyces* strains that most likely can synthesize γ-butyrolactones with affinity toward ScbR.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions

Streptomyces strains M145, LW16 (this work), and *ΔscbA* (M751; Takano et al., 2001) were manipulated as described elsewhere (Kieser et al., 2000). *E. coli* JM101 was manipulated according to Sambrook et al. (1989). MS agar (Kieser et al., 2000) was used to prepare spores and selection of exconjugants. SMM and SMMS agar (Takano et al., 2001) was used to isolate γ-butyrolactones. SMMS agar (Takano et al., 2001) was used for the antibiotic bioassay. DNAgar containing 5 μg/ml kanamycin was used for the kanamycin bioassay. pGEM-T (Promega) was used for cloning polymerase chain reaction (PCR) products. pIJ487 (Ward et al., 1986), pIJ6120 (Takano et al., 2001), and pIJ82 (Hong et al., 2004) were used to construct the new reporter.

Purification and Structural Determination of γ-Butyrolactones

The purification was conducted as reported previously (Takano et al., 2000). The HPLC peak samples with precocious antibiotic production from these purification was further analyzed by using a C18 reverse column (SHISEIDO C18 capcellpak SG120/5 μm, 4.6 mm × 250 mm) with a UV detector (Nippon Bunko UVIDEC-100V) with elution solvent of 30% CH_3CN/70% H_2O/ 0.1% TFA and flow rate of 1.0 ml/min.

^{1}H-NMR analysis was done on a Nippon Denshi model JNM-GSX-400 spectrometer at 400 MHz in $CDCl_3$ using $CHCl_3$ δ_H 7.25 as an internal reference.

^{1}H-NMR data for 2-(1′-hydroxyoctyl)-3-hydroxymethylbutanolide: δ 4.43 (1H, *t*, J = 8.8 Hz, H-4a), 4.02 (1H, *m*, H-1′), 3.99 (1H, *t*, J = 8.8 Hz, H-4b), 3.76 (1H, *dd*, J = 5.6, 10.6 Hz, H-5a), 3.70 (1H, *dd*, J = 5.6, 10.6 Hz, H-5b), 2.78 (1H, *m*, H-3), 2.66 (1H, *dd*, J = 4.8, 9.2 Hz, H-2), 1.26∼1.30 (10H, *m*, H-3′∼H-7′), and 0.89 (3H, *t*, J = 6.5 Hz, H-8′). One-dimensional 400 MHz ^{1}H-NMR (Figure S2) indicated that the purified compound possessed a typical 2,3-disubstituted γ-butyrolactone skeleton, as evident from the signals at 4.43, 4.02, 3.99, 3.76, 3.70, 2.78, and 2.66 ppm (see detailed explanation in Figure S2).

Nano LC-Orbitrap MS Analysis of SCB1, SCB2, and SCB3

SMMS grown M145 ethyl acetate extracts resuspended in 100% MeOH was analyzed using nano-liquid chromatography (LC) on-line coupled to an Orbitrap-MS (Thermo Scientific Corporation). Chemically synthesized SCB1, SCB2, SCB3, and IM-2 $C_{9\text{-i}}$ (1 ng/μl) were used as reference standards and SMMS and *ΔscbA* ethyl acetate extracts were injected as negative control.

The system was operated with electrospray ionization source in positive mode. LC conditions were as follows: the column was a Dionex C18 column (Acclaim PepMap100, C18, 3 μm, 75 μm i.d. × 15 cm); the mobile phase consisted of A (H_2O/0.1% formic acid) and B (acetonitrile/0.1% formic acid); the gradient elution started with 20% B at 3 min, 95% B at 45 min, 95% B at 50 min, and 20% B at 55 min and was then kept at 20% B until 60 min, flow rate 0.200 μl/min, MS mass detection range 150–500 Da. One microliter of the reference standards and 5 μl ethyl acetate extracts were injected. Analysis was done by using Xcalibur software (Thermo Fisher Scientific).

Isolation of γ-Butyrolactones and the Antibiotic Bioassay

γ-Butyrolactone isolation from liquid or solid cultures and the antibiotic bioassay were followed as described previously (Takano et al., 2000).

Construction of *scbA* and *scbR* Double Deletion Mutant

To construct the *scbA scbR* double deletion mutant, the disruption cassette, *aac(3)IV*-oriT, from the plasmid pIJ773 (Gust et al., 2003) was amplified by the primers, RB4F and RB4R (Supplemental Data) and the resulting PCR product was cloned into the cosmid AH10 and then introduced into *S. coelicolor* according to the REDIRECT procedure (Gust et al., 2003). M145 recombinants were screened for apramycin resistance and the deletion was confirmed by PCR using the primers DDAR2 and scbA sec2 (Supplemental Data), and by Southern analysis (data not shown). The mutant was named *S. coelicolor* LW16.

Construction of the Kanamycin Assay Strain

The promoterless kanamycin gene was PCR amplified from the plasmid pIJ487 by using the primers Tnneo1 and neo2 (Supplemental Data). The PCR fragment was cloned into pGEM-T vector (Promega) to obtain pTE130. The *Eco*RI digested fragment from pTE130 was subcloned into the *Eco*RI site of pIJ6120 (which contains an intact *scbR* and its own promoter region) in the orientation so that the start codon of the promoterless kanamycin gene is flanked by the *Sac*I site (Takano et al., 2005), to create pTE131. A 146 bp PCR fragment, corresponding to −25 to +34 from the starting site, containing a *cpkO* promoter and the ScbR binding site region (Takano et al., 2005) was amplified by using the primers pkasO1 and pkasO2 (Supplemental Data), which contain an *Sac*I restriction site at the 5′ end of each primer, and *S. coelicolor* M145 genomic DNA was used as template. After digestion with *Sac*I, the 137 bp PCR fragment was ligated into *Sac*I-digested pTE131 with the promoter orientated to direct transcription toward the promoterless kanamycin gene to obtain pTE133. A 2.22 kb BglII fragment from pTE133 was then subcloned into a BamHI site of pIJ82, an integrating vector, to yield pTE134. The relative orientation of the *scbR* and the *cpkO* promoter with the *neo* was confirmed by PCR with the primers pkasO1, pkasO2, scbRrt2, and neo2. pTE134 DNA was sequenced by MWG Biotech AG (data not shown).

pTE134 was introduced into the methylation-deficient *E. coli* strain ET12567 containing the RP4 derivative pUZ8002 (Flett et al., 1997), and transferred to *S. coelicolor* LW16 by conjugation (Flett et al., 1997). Single-crossover exconjugants were selected on MS containing hygromycin and nalidixic acid, to obtain transconjugants LW16::pTE134. The genomic DNA was isolated and plasmid integration was confirmed by PCR with the primers JGattB1-fwd and JGattPint-rev (Supplemental Data), which gave an amplified product of 0.8 kb corresponding to the inserted region (data not shown).

The Kanamycin Bioassay

Chemically synthesized autoregulator analogs or methanol-extracted γ-butyrolactones from *S. coelicolor* M145, *ΔscbA*, and various *Streptomyces* species

were spotted onto a confluent lawn of *S. coelicolor* LW16::pTE134 spores plated on DNAgar supplemented with kanamycin at a final concentration of 5 μg/ml and then incubated at 30°C for 3 days. Methanol was used as a negative control. For detailed protocol see Hsiao et al. (2009). To determine the detection range of the kanamycin bioassay, 62.5 μg to 0.01 μg autoregulator analogs was tested.

Chemical Synthesis of Autoregulator Analogs

A-factor type analogs (Figure 4) containing a C-1′ keto group with various alkyl side chains at C-2 were synthesized as previously described (Nihira et al., 1988) by reacting 3-(trimethylsilyoxymethyl)butanolide with a suitable alkyl chloride.

Virginiae butanolide (VB) type analogs (Figure 4) and IM-2 type analogs (Figure 4) with various linear alkyl side chains at C-2 were synthesized as described before (Kim et al., 1989) by reacting 3-(trimethylsilyoxymethyl)butanolide with a suitable linear aldehyde by Aldol condensation.

For the synthesis of VB-type and IM-2 type analogs containing 1′-hydroxy-7′-methyloctyl, 1′-hydroxy-6′-methyloctyl, or 1′-hydroxy-5′-methylheptyl side chain (VB $C_{9\text{-i}}$, IM-2 $C_{9\text{-i}}$, VB $C_{9\ s}$, IM-2 $C_{9\ s}$, VB $C_{8\ s}$, or IM-2 $C_{8\ s}$), corresponding branched-chain carboxylic acids (7-methyloctanoic acid, and 6-methyoctanoic acid) were first synthesized from 5-bromovaleric acid and isobutyl bromide or *sec*-butyl bromide, respectively, using modified Grignard reaction in the presence of dilithium tetrachlorocuprate (Baer and Carney, 1976). Alkyl chloride was prepared from the branched-chain carboxylic acid and was used to synthesize the corresponding A-factor-type analogs, and reduction with NaBH4 gave corresponding VB-type and IM-2-type analogs (Figure 4).

SCB1 and its isomers (Figure 1), which contain 1′-hydroxy-7′-methylheptyl side chain at C-2, were synthesized as previously described (Takano et al., 2000).

The synthesized compounds were purified by C18 HPLC and the structure confirmed by ^{1}H-NMR analysis on a Nippon Denshi model JNM-GSX-400 spectrometer at 400 MHz in $CDCl_3$ using $CHCl_3$ δ_H 7.25 as an internal reference.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and nine figures and can be found with the article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00279-8.

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